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優勝作品專輯

作品編號　060005

參展科別　植物學

作品名稱　隱密的發育調節中樞-植物轉錄因子 BPC 對發育之調控機制 A cryptic hub for development control: Unraveling the regulatory role of plant transcription factor class I BASIC PENTACYSTEINEs in Arabidopsis development

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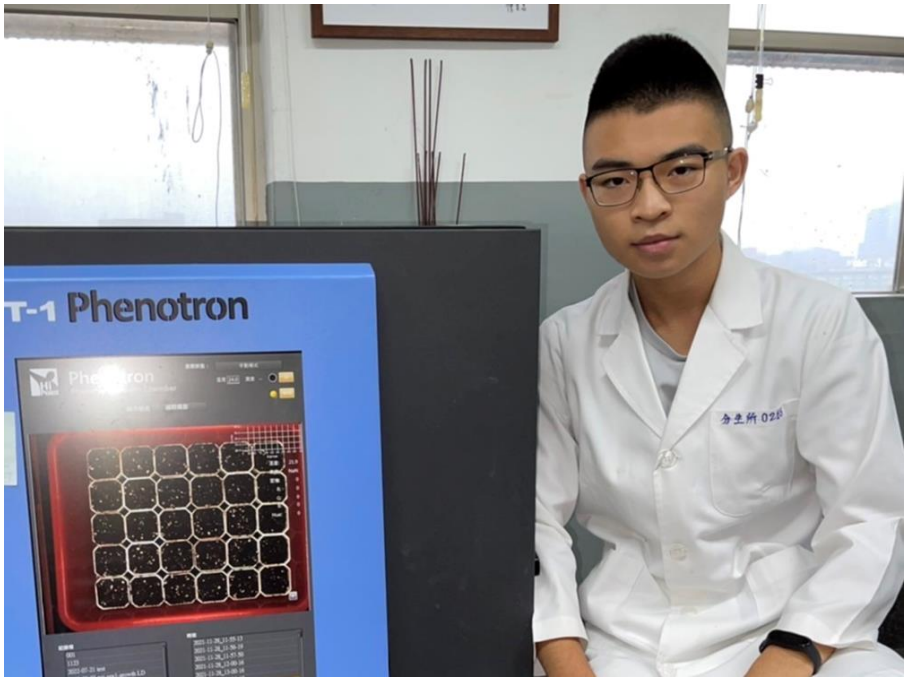

我是蔡紹騏，現就讀建國中學數理資優班二年級。面對學界未知的問題，沒有教科書的解答，只能憑藉自己的實驗設計與操作毅力解惑。我很慶幸能在高中進行專題研究，跳脫了選填記誦的學習模式，提早培養於學術前緣鑿採所需具備的研究素養及論辯思維。感謝父母支持我對生物學的熱忱，也感激蔡皇龍教授、朱芳琳老師與李宜臻學姐一路來的指引和教導。

摘要

GAGA 序列為生物發育重要順式作用子； BPC (BASIC PENTACYSTEINE) 則為植物特有 GAGA 結合蛋白。已知 *bpc* 突變體具多效性，其生理時鐘相關之發育有多重缺陷。阿拉伯芥 BPC 家族中 BPC1, BPC2, BPC3 為第一亞群，且 BPC 群間和群內有重疊與拮抗作用。為探究第一群 BPC 是否調控生理時鐘，本實驗以 3D 影像觀察 *bpc1 bpc2*、*bpc1 bpc2 bpc3* 及野生型之晝夜運動，並誘導 BPC 過量表現以檢測時鐘基因反應，發現 *bpc* 突變體之晝夜運動與時鐘節律皆有缺陷，顯示 BPC 能影響生理時鐘運行。透過一系列對第一群 BPC 突變體與過量表現植株的 RT-qPCR 檢測，可歸結第一群 BPC 是能調控生理時鐘與葉片生長的中心。

Abstract

GAGA motifs are found upstream of various genes regulating plant growth, and BPCs (BASIC PENTACYSTEINEs) are plant-specific GAGA-binding factors. Past research demonstrated that *bpc*s are pleiotropic mutants, leading to multiple developmental defects relating to the circadian clock. BPC1, BPC2, and BPC3 can be categorized into class 1, BPC4, BPC5, and BPC6 into class 2 respectively, with BPC5 being a pseudogene. It was proved that between and within classes, BPCs have overlapping and antagonistic regulations. We aim to analyze how class 1 BPCs regulate the circadian clock of *Arabidopsis thaliana*. By monitoring the leaf circadian motion via 3D point clouds and implementing overexpression of BPCs with an inducible system. We compared downstream clock operation in double *bpc1 bpc2* and triple *bpc1 bpc2 bpc3* mutants with WT. The leaf motion was restricted in the *bpc* mutants, suggesting that clock operation was compromised. By conducting a series of RT-qPCR assays, we examined clock gene expression under mutant and induced lines of class I BPC. Our results showed that class I BPCs are interlocked to form a repressive machinery repressing or activating the essential genes of the circadian clock and leaf development. To conclude, it can be said that class I BPC function as a regulatory hub towards the circadian clock and leaf development.

Research Motivation

The regulation of plant development is vital for environmental fitness to survive and propagate. Broadening our understanding of plant development will help in breeding functional crops. The developmental processes are sophisticatedly regulated in eukaryotic multicellular organisms. The developmental processes are sophisticatedly regulated in eukaryotic multicellular organisms. In plants and animals, GA/TC-dinucleotide (GAGA) repeats in varied patterns were frequently found in the upstream DNA of structure genes crucial for growth and organogenesis [1-6]. The GAGA-element in regulating the development and growth is preserved across animal and planta and recruits polycomb repressive complexes (PRCs) for constituting the dynamic modification of the epigenome of given organisms [4,6-10]. Even though the cis-element system is preserved for developmental gene regulation in animals and plants, the trans-acting factors utilizing the GAGA-element for PRC recruitment are evolutionarily divided. The family members of BASIC PENTACYSTEINE (BPC) are plant-specific transcription factor binding to the GAGA motifs [11]. BPC members constitute a complex gene network with overlapping and antagonistic functions between the same and different subclasses of family members [1,4,9,10,12,13]. To further elucidate the co-functional mechanism of BPC members, we deciphered the role of class I BPCs on plant circadian growth and clock regulation in *Arabidopsis thaliana*. This knowledge can be further applied to generate strategies for tuning the flowering time and leaf shape of ornamental plants or economic crops.

Research Background

The impact of the BASIC PENTACYSTEINE family on the plant development

Gene expression is the fundamental molecular basis for proper growth and development in plants and animals. GA/TC-dinucleotide repeats (GAGA-motifs) are

regularly found upstream of homeotic genes for organism development. GAGA-motif binding proteins are naturally transcription factors controlling the development processes; nevertheless, so far there are no homologous proteins found in GAGA-binding between plants and animals. The BARLEY B RECOMBINANT/BASIC PENTACYSTEINE (BBR/BPC) family is composed of plant-specific GAGA-binding proteins [11]. It has been revealed that multiple developmental pathways are deficient in mutants lacking BPCs in various combinations. BPC members conduct overlapping and antagonistic functions simultaneously on hypocotyl elongation, rosette extension, and flowering time [12,14-16], which are controlled by the phytohormones and environmental cues. In Arabidopsis, BPC transcription factors are categorized into three subclasses, class I: BPC1, BPC2, and BPC3; class II: BPC4, BPC5, and BPC6; and class III: BPC7 [11]. BPC5 is a putative pseudogene harboring a premature stop codon in its coding region [12]. The other members of class I and II BPCs constitute a repressive network for developmental gene repression [17]. Simultaneously, additively repress the transcription of *BPC3*, of which the ectopic expression causes severe defects by impeding leaf edge and reproductive organ development [12,14]. The vegetative development of mutants carrying BPC3 without other class I and II BPCs are drastically retarded [12]. Such retardation is partially rescued by introducing the *BPC3* mutation [12], indicating that *BPC3* functions as a cryptic regulator harmful to plant development during the vegetative phase.

Besides the antagonistic function between BPC3 and other BPCs, BPC3 bears an overlapping function with BPC1 and BPC2 on shoot apical meristem size maintenance [18], lacking BPC class I members enlarges inflorescence meristem and increases flower number [12,18]. All three BPC class I members are capable of recruiting FERTILIZATION-INDEPENDENT SEED-Polycomb Repressive Complex2 (FIS-PRC2) to mediate proper development spanning prezygotic to postzygotic stages within pistil [17]. This suggests that BPC3 functions with other class I BPCs to regulate apex-specific organogenesis and

vegetative-to-reproductive transition [17,18]. The genetic and functional assays on BPCs have revealed that the BPC family is spatiotemporally tuned to conduct a sophisticated regulation of homeostatic genes during development.

BPCs are involved in the circadian clock regulation in Arabidopsis

Individuals in a population of plants are sessile and adapt themselves to the changing conditions in the environment. A pivotal strategy for plant survival across seasonal changes is accomplishing the alternation of a generation before or after the winter, which generally causes chilling injury to plants in high-latitude regions. Most Arabidopsis accessions used in laboratories are summer-annual and flowering depending on the external long-day photoperiod and/or on their vegetative maturity autonomously. The photoperiodic and autonomous pathways are rhythmic mechanisms for plants to cycle their developmental processes at periodic domains spanning from day to season. Though the two pathways constitute the chronobiological system assisting plants in flowering at a suitable time, the system can be vulnerable once it overreacts to unexpected environmental changes. Therefore, flowering control pathways are sustained by the internal oscillation system, the circadian clock, which keeps rhythmic pathways stay in the right track constituted by a central oscillator system [19-24].

In Arabidopsis, the backbone of the central oscillator is first described by *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*)*/LATE ELONGATED HYPOCOTYL* (*LHY*) and *TIMING OF CAB EXPRESSION1* (*TOC1*), the representative dawn and evening genes repress the expression of each other at the corresponding time during a day [25,26]. The expression of *CCA1/LHY* is increased gradually before and reaches its peak at dawn. Oppositely, the expression of TOC1 is initiated during the evening and reaches its peak at midnight [25]. Other clock components are expressed between the peaks of *CCA1* and *TOC1*, *PSEUDORESPONSE REGULATOR9* (*PRR9*), *PRR7*, and *PRR5* peak from morning to afternoon and repress *CCA1/LHY* consecutively with *TOC1* [27-29]. The protein level of TOC1

in the PRR-repressive system is down-regulated by ZEITLUPE (ZTL), an E3 ubiquitin ligase, which is stabilized post-translationally before dusk by the evening component GIGANTEA (GI)-recruited deubiquitinases [30]. Besides the transcriptional repressors, positive activators are also found to be involved in clock regulation. The two co-activator systems formed by *LIGHT-REGULATED WDs* (*LWDs*) and class I *TEOSINTE BRANCHED1, CYCLOIDEA, PROLIFERATING CELL FACTORS20/22* (*TCP20/22*)[31-33], and by *NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATEDs* (*LNKs*) and myb-like *REVEILLEs* (*RVEs*)[34-36] activate *CCA1* and *TOC1* at dawn and night, respectively. The positive-negative forces may have secured the flexibility and robustness of clock oscillation under the day-night cycle in which the environment is usually accompanied by unpredictable random changes.

A clock gene is not only connected with the central oscillator system by the transcription-transcription feedback loop contributed by other clock components. A suite of clock genes and downstream targets harboring overrepresented cis-elements of GAGA-motifs has been identified [37], implying that GAGA-binding factors may be involved in the clock regulation. The GAGA-motifs found upstream clock genes are consistent with the pleiotropic defects shown by the high-order mutants of *bpc1-1 bpc2 bpc4 bpc6*, which is retarded in circadian expansion of rosette region [14]. In addition, the ectopic expression of BPC3 inhibited the expression levels of multi-clock genes and stalhe clock oscillation [14]. This indicates the BPC-directed repressive machinery is involved in the clock regulation.

Research Purpose

Even though the genetic effects of BPC family are unraveled on multiple developmental processes in Arabidopsis [12,14], the molecular mechanisms that an individual member in the BPC family communicates with other members and developmental genes are rarely addressed. In this work, we focused on investigating the regulatory roles of the class I BPC members in the vegetative development. We analyzed the leaf-motion and

related genes in lose-of-function mutant combinations. In addition, we unraveled the connection between developmental phenotypes and molecular mechanisms in a series of inducible lines of gain-of-function class I BPCs. Our work gains insights that class I BPCs form a cryptic hub regulating circadian clock and leaf edge development in Arabidopsis.

Material and Methods

Plant material

- Columbia-0 (Col-0)
- *bpc1-1 bpc2* (*bpc1,2*) (CS68700)
- *bpc1-1 bpc2 bpc3-1* (*bpc1,2,3*) (CS68699)
- pER8: BPC1-EYFP-HA/ Col-0
- pER8: BPC2-EYFP-HA/ Col-0
- pER8: BPC3-EYFP-HA/ Col-0

The origin of the *bpc1-1* mutant is Salk_072966, inserted with T-DNA, located 232 bp upstream of ATG; the origin of the *bpc2* mutant is Salk_090810, inserted with T-DNA, located at K72; the origin of the *bpc3-1* mutant is TILLING, of which SNPs located at Q230 lead to a putative stop codon before exon-exon junctions, potentially causing nonsense-mediated mRNA decay.

Phytoagar plate preparation

Agar plates are needed for the early stages of seed growth. The formula for the plates is listed in the table below:

1/2 MS medium	
MS salts with vitamins (CaissonLabs)	4.43 g
Deionized H2O	1800 mL
Adjust pH to 5.7 with 1N KOH	~800 **µ**L

Bring the volume to 2L with deionized water	
0.8% phytoagar (Duchefa Biochemie)	16 g

Autoclave the medium mixture at 121 °C under 15 psi pressure for 15 minutes Distributed the autoclaved medium at about 55 °C into 9 cm petri dish (2 cm high), cooled at room temperature for solidification, store at 4 °C until used.

Seed sowing

For both mutant and overexpression lines, we conducted the same method of seed sowing on phytoagar plates, which included the following steps:

1. Put the agar plates to dry in linear flow.
2. Transfer the seeds to a 1.5 mL centrifuge tube with clean paper sheets.
3. Add 300 μL of bleach solution containing 1.5% sodium hypochlorite and 0.025% Sodium dodecyl sulfate (SDS) to the tube via a pipetman.
4. Flip the tube for 3 minutes to make sure all the seeds are infiltrated with the bleach solution. (Do not let the seeds be in contact with bleach for too long to avoid damage)

The following procedures should be conducted in linear flow to avoid contamination.

5. Remove the bleach solution with a p1000 pipetman.
6. Add 300 μL of dH2O to wash up the seeds. Flip the tube multiple times.
7. Remove the dH2O. (Be cautious not to suck up the seeds)
8. Repeat steps 6 and 7 to make sure all the bleach is washed away.
9. After adding 300 μL of dH2O to the tube, draw lines at the bottom of the agar plates to divide sections for different types of seeds and label the sections.
10. Gently place the seeds one by one onto the plate with a p100 pipetman. Seeds are to be sowed with adequate growth space.

After sowing is completed, seal the petri dish with paper tape and place the plate into the growth chamber.

Capturing of leaf motion

We track the leaf movement of *Arabidopsis* plants, Col-0, *bpc1*,*2*, and *bpc1*,*2*,*3*, via a 3-dimensional (3D) binocular camera every hour (Figure 1A). Seeds after sowing were put into a growth chamber to grow under long-day conditions for fourteen days (16-h light/8-h dark, 80-100 μmol m^{-2} s^{-1} of white light). Then the seedlings were transf erred to 5cm*5cm*5cm pots containing a soil mixture of Jiffy peat, pearlite, vermiculite, and water mixed to the volume proportion of 9:1:1:1. For the next 3 days, the seedlings were monitored by recording the elevation angle at the indicated time under the day-night cycle.

The distance between the center of the camera's lens was measured to be 7.5 centimeters, and the distance between the lens and the top of the pots was 37.5

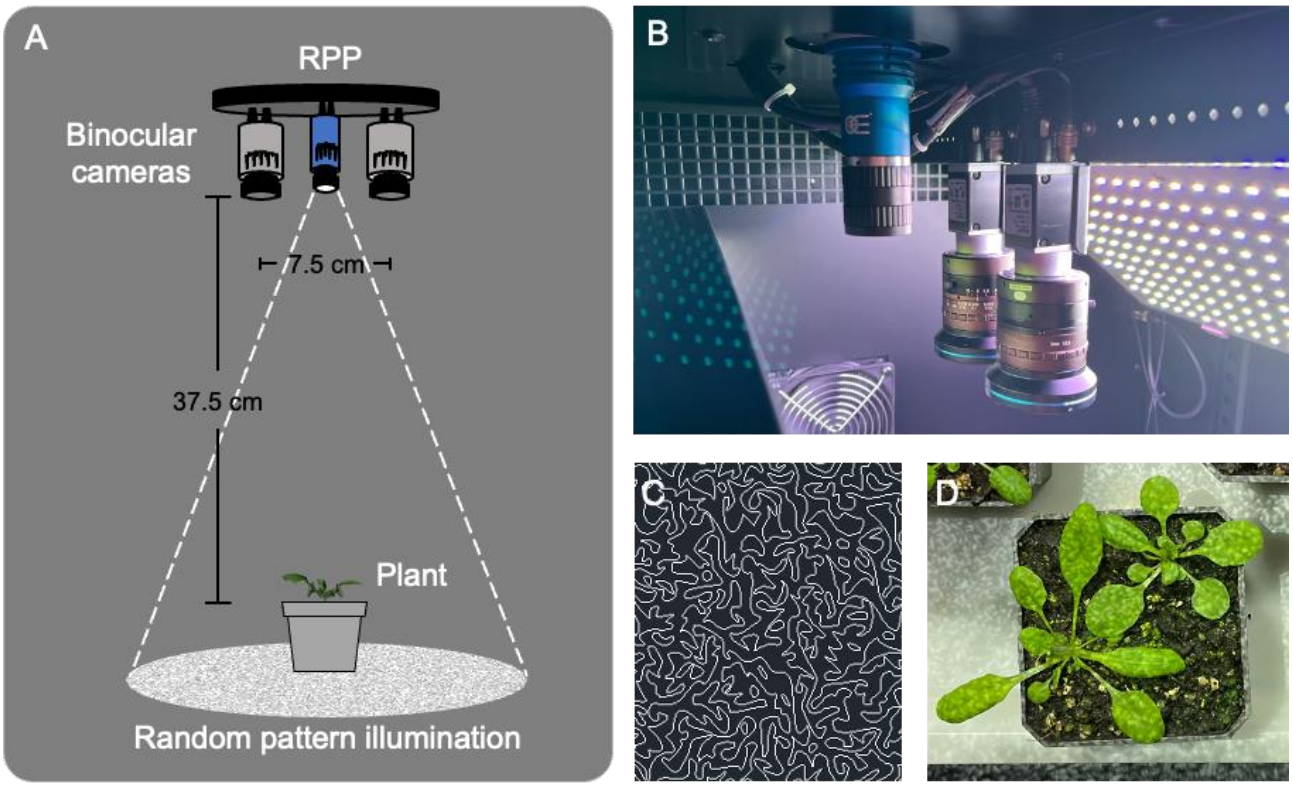

Figure 1 | The 3D image of a sample plant is obtained by the binocular camera

(A) The active stereo vision assembled in the growth chamber is illustrated. The distance between the two lens centers of the binocular cameras is 7.5 cm. The camera height is 37.5 cm from the baseline of the plant. (B) The actual equipment is shown. The random pattern projector (RPP) illuminates the plant with a randomly grated white light when the binocular cameras image the plant. (C) The random grate of the RPP. (D) Plants are imaged by illuminating the random pattern light.

centimeters (Figure 1A and 1B). A projector casted random arrangement of light and dark spots onto the platform bearing the soil pots as reference points (Figure 1C and 1D). After all procedures were carried out, the data (x,y,z coordinates and R,G,B values) was inputted into the software, CloudCompare, where they constructed point cloud simulation of the seedlings.

RNA extraction

After 18 days of growth under long-day conditions, the seedlings were transferred to constant light (LL) for free the running of the circadian clock. The seedlings under the free-running condition were harvested every 3 hours starting from the 24th to 72nd hour of lasting light conditions. Five Seedlings were quenched by liquid nitrogen in a 2.0 mL centrifuge tube and stored in the -80-degrees-Celsius refrigerator until used. Total RNA was extracted from the seedlings with the pine-tree method, which consists of these following steps:

1. Place five of 2.3 mm stainless steel beads into each tube. (Do not let the tube leave liquid nitrogen for too long to keep the temperature low)
2. Preheat the pine-tree buffer in a bath to 65 degrees Celsius with an adequate amount of R.O. water to well conduct the heat.
3. Grind the seedlings into fine powder with a homogenizer. (It is suggested that the timer of the homogenizer be set to 10 seconds and repeat the homogenizing for 15 times. Between each repeat, dip the tubes back into liquid nitrogen to keep the samples at low temperatures)
4. Add the pre-heated pine-tree buffer to the tubes. I applied 700 μL minimum volume of buffer to sample powder up to 100 mg. (The powder could potentially burst out of the tube due to contact with the room-temperature buffer liquid. Hence, the adding of the buffer must be as gently as possible)
5. Vortex the tubes vigorously to ensure the powder is completely infiltrated with the buffer. Incubate the samples at 65 degrees Celsius for 5 minutes.

6. Briefly spin down and transfer the mixture to a new set of 1.5 mL centrifuge tubes to remove the stainless-steel beads.
7. Add 700 μL of chloroform into the tubes in hood. Vigorously shake the tubes so that the mixture is well mixed.
8. Put the tubes into a centrifuge and spin under 4 degrees Celsius at 15000 rpm.
9. Save the aqueous phase in a new 1.5 mL tube and bring the final concentration of LiCl to 2 M by using 10 M LiCl solution.
10. Put the mixture in a 4-degrees-Celsius Refrigerator overnight to precipitate the RNA.
11. Put the tubes into a centrifuge and spin under 4 degrees Celsius at 15000 rpm the next morning. Add 800 μL of 75% ethanol after removing the supernatant. Flip the tubes multiple times until the pellets suspend to cleanse the pellets.
12. Once again put the tubes into a centrifuge and spin under 4 degrees Celsius at 15000 rpm, and then remove the residue ethanol.
13. Briefly air dry the pellet and suspend the RNA pellet in 25 μL of DEPC-treated H2O on ice.
14. Read the ratio of absorbance at 260 nm and 280 nm to assess the purity and concentration of RNA of each tube using a micro-spectrometer (Nano-500, MEd Club Scientific Co., LTD).
15. Dilute each tube to a concentration of 250 ng/μL, and then take 8 μL (2 μg total RNA) for reverse transcription. (Doing such can decrease pipetting error)
16. Save RNA samples in the minus-80-degrees-Celsius refrigerator for long-term storage.

Pine-tree Buffer	
Reagent	**Concentration**

CTAB (hexadecyltrimethylammonium bromide)	2%
PVP (polyvinylpyrrolidone K 30)	2%
EDTA	25 mM
NaCl	2.0 M
spermidine	0.5 g/L
Autoclave for 20 minutes.	
Tris-HCl pH 8.0	100 mM
beta-mercaptoethanol (add just before use)	2%

Chloroform	
Chloroform: isoamyl alcohol	24:1

DEPC-H2O
Dissolve 1 ml diethylpyrocarbonate (DEPC) in 1L ultra-pure water and incubate at 37°C overnight. Autoclave for 40 minutes.

Reverse transcription

Two micrograms of total RNA were used for synthesis of the first strand complementary DNA (cDNA) in the reverse transcription (RT) using SuperScript IV (SSIV) reverse transcriptase kit (Cat. No. 18090050, Thermo Fisher).

1. Cleanse the RNA sample with DNase I. Use 2 units for 2 μg total RNA in a 10-μL reaction (Cat. No. M03033, New England Biolabs). Incubate at 37 degrees Celcius for 30 minutes.
2. Add Oligo(dT)$_{23}$(dA/dC/dG) primer (purchased fsorm Integrated DNA Technologies) to a final concentration of 2.5 **μ**M. Add 4dNTP each to a final concentration of 0.5 mM bring the total volume to 13 **μ**L by DEPC-treated ddH2O. Incubate the mixture at 65 degrees Celsius for 5 minutes and quench it on ice for at least 1 minute.
3. Finalized the RT reaction by adding 0.5 **μ**L DEPC-treated ddH2O, 4 **μ**L 5x SSIV buffer, 1 **μ**L 100 mM DTT, 1 **μ**L RNase inhibitor (Cat. No. N251B, Promega), and 0.5 **μ**L SSIV reverse transcriptase (200 units/**μ**L; Cat. No. 18090010, Invitrogen). Incubate the RT reaction at 55 degrees Celsius for 10 minutes and inactivate the reverse transcriptase at 80 degrees Celsius for 10 minutes. Dilute the synthesized cDNA 20 times with ddH2O and store it at -20 degrees Celsius until used.

qPCR

qPCR Mixture		
Reagent	**Amount for 100 tubes (μL)**	**Amount per tube (μL)**
2* qPCRBIO SyGreen Mix	700	7
Forward Primer (10**μ**M)	4.5	0.045
Reverse Primer (10**μ**M)	4.5	0.045
ddH2O	491	4.91
Total a1mount	1200	12

After adding 12 μL of mixture and 2 μL of cDNA to 8-stripped 0.2 mL tubes (cDNA should be well mixed before added to the tubes), spin down the solution and load it onto the qPCR machine. The qPCR protocol is shown in Figure 2.

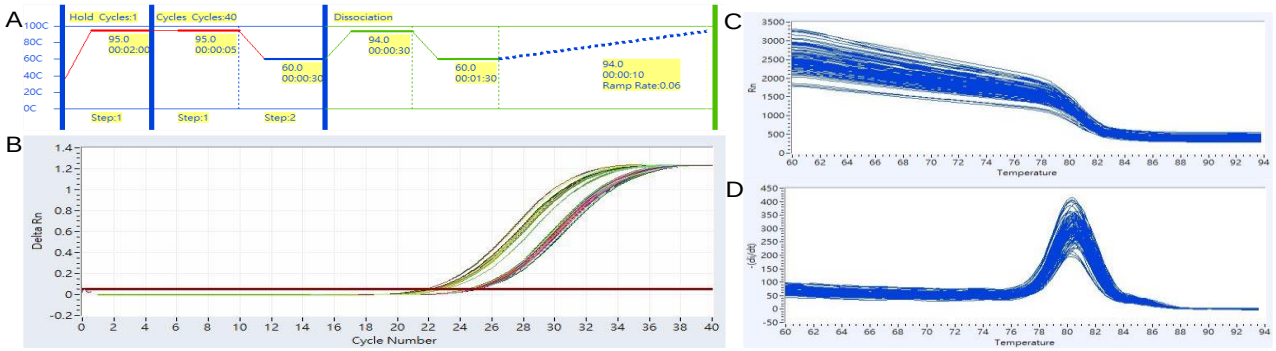

Figure 2 | *qPCR program and plot diagram*

(A) The qPCR program is divided into three parts, separated by the two vertical blue lines in the diagram. Part 1 lasts for 2 minutes, heating up to 95 degrees Celsius to dissociate the dsDNA entirely. Part two is an amplification cycle of 95 degrees for 5 seconds and 60 degrees for 30 seconds, repeating forty times. Part 3 is to determine the melting curve. After heating up to 94 degrees Celsius for 30 seconds then cooling down to 60 degrees for 90 seconds, it heats up gradually at the ramp rate of 0.06 degrees Celsius per 10 seconds. (B) The amplification results from part 2 of the program is shown in this diagram. Threshold is set at 0.05. (C) The melting curve from part 3 of the program is shown in this diagram. (D) The positive value of the slopes of each melting curve in (C) is drawn in this diagram. In theory, the template strands of a target mRNA is determined by sequence and length, resulting in the same Tm value (The temperature at which florescence intensity drops to half). Through (D), we can check if there are any unwanted primer-dimers or nonspecific amplicons.

Western Blotting

The seedlings were collected after two-day induction with 0- or 50-mM β-ES treatment under LL. Seedling tissue was frozen in liquid nitrogen, and then ground with a pestle into powder. Sample powder was lysed in 50 μL of pre-heated 2x Laemmli sample buffer (0.12 M Tris-HCl pH 6.8, 2% β-mercaptoethanol, 2% SDS, 20% glycerol, 0.02% bromophenol blue). The mixture was incubated at 95 degrees Celsius for 5 minutes and centrifuged 14,000 rcf for 14 minutes at room temperature. Afterwards, the supernatant

was transferred to a new set of Eppendorf 1.5 mL tubes. Western blot analysis was conducted afterwards following the proceeding steps:

1. Cleanse the glass and porcelain planks with 75% ethanol before securing them onto the gel cassette.
2. Prepare the separation gel and stacking gel solutions

separation gel (10%)		stacking gel (5%)	
30% Acrylamide	1.5 mL	30% Acrylamide	200 µL
1.5 M pH8.9 Tris	1.125 mL	1.5M pH8.9 Tris	150 µL
ddH_2O	1.875 mL	ddH_2O	0.85 mL
10% APS	45 µL	10% APS	12.5 µL
TEMED	4.5 µL	TEMED	1.25 µL

3. Load the protein markers along with the protein extracts into the SDS-gel in 1x Tris-Glycine running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). Run at 120 volts for 15 minutes (in stacking gel) and at 150 volts for 45 minutes (separation gel).
4. Pre-activate a Polyvinylidene difluoride (PVDF) membrane with 95% ethanol. Place the membrane, the SDS-PAGE, and two pieces of 3M filter paper into the transfer tank system. Be sure to face the gel towards the negative pole and the membrane towards the positive pole respectively. Perform the protein transfer in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol) on ice at 80 volts for 90 minutes.
5. Prepare 0.25% skim milk (0.25 g skim milk in every 100 mL 1x PBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.1% Tween® 20 detergent)
6. Prepare the primary antibody mixture (0.625 µL mouse-anti-HA (Sigma-Aldrich) in 5 mL milk per piece of PVDF membrane)
7. Prepare the secondary antibody mixture (0.2 µL mouse-anti-HA (Sigma-Aldrich) in 5 mL milk per piece of PVDF membrane)
8. Place the membrane upright inside a holder.

9. Conduct the immunoblotting with SNAP i.d. ® 2.0 Protein Detection System; block with 30 mL 0.25% slim milk per slice of PVDF membrane.
10. Add 5 mL of primary antibody mixture and incubate for 10 minutes at room temperature. Afterwards, wash three times with the same mixture. Wash four times with 30 mL 1x PBST.
11. Add 5 mL of secondary antibody mixture and incubate for 10 minutes at room temperature. Afterwards, wash three times with the same mixture. Wash four times with 1x PBST.
12. With immunoblotting conducted, place the membrane onto transparent plastic backing plates to drain. Add 50 µL each of Luminol/ Enhancer buffer and Peroxide buffer (SuperSignal™ West Femto Maximum Sensitivity Substrate) to the membrane and spread it evenly with another plastic plate. Use a luminescence imaging system to detect the signal under 30s exposure (or for appropriate time).
13. Stain the membrane with 0.1% Coomassie blue (50% methanol, 10% acetic acid, 0.1% Coomassie Brilliant Blue R250) for 10 minutes with a shaker. Wash off excessive stain with R.O. H_2O. Scan the membrane for loading control.

Results

The circadian motion of mutants lacking class I BPCs is impeded

It has been shown that mutants deficient in BPC family members are pleiotropic in multiple phenotypes related to the circadian clock, including hypocotyl and petiole elongation, rosette expansion, and flowering time. Therefore, we further recorded the leaf movement of class I BPC mutants to characterize circadian motion. The plants of the wild type Columbia-0 (Col-0), *bpc1-1 bpc2* (*bpc1,2*), and *bpc1-1 bpc2 bpc3* (*bpc1,2,3*) were monitored by 3-dimensional (3D) binocular camera every hour, which obtained the depth information of pixels in the 2D image. However, some pixels in the steep leaves were undetectable. We

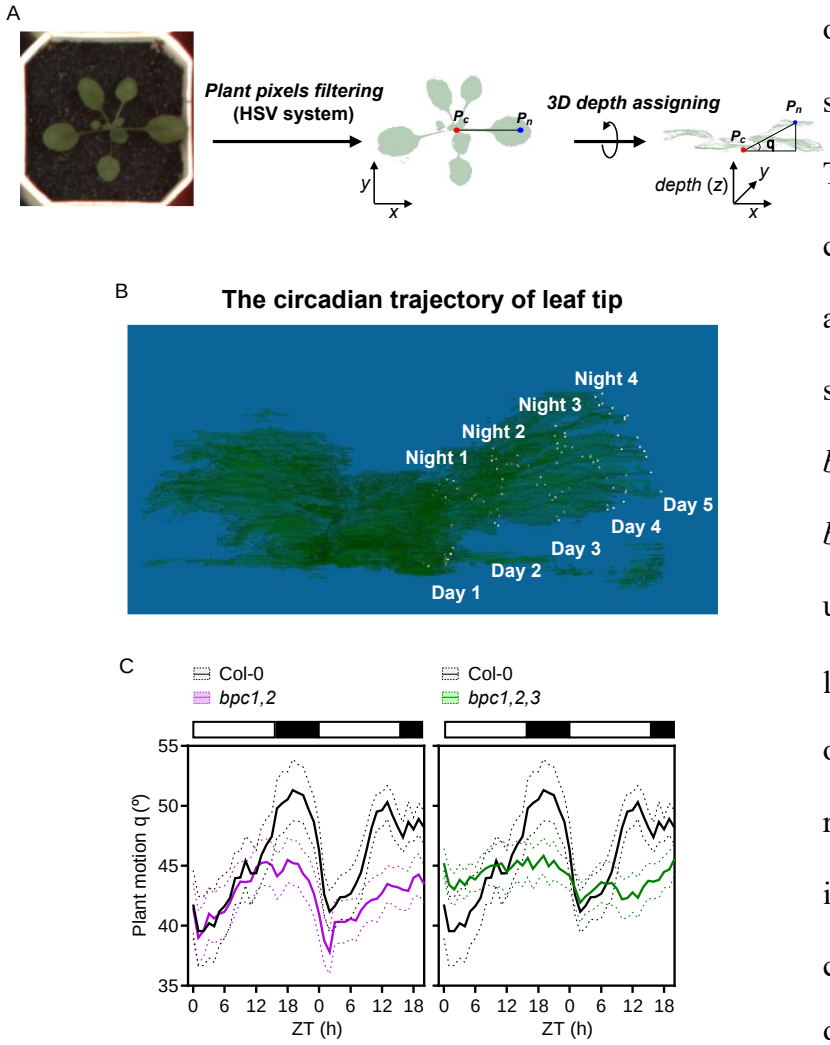

Figure 3 | The circadian motion of *bpc* mutants is impeded

(A) The 2D image of the representative Arabidopsis (Col-0) was color-filtered to extract plant pixels by using the HSV color model (pixels passed criteria of hue:50-175 and value:30-60 were preserved). The plant pixels were further processed by assigning depth information from a 3D camera. The central pixel of the plant in the *xy* plane was manually selected and assigned with the lowest depth value (z) to define as the basal point (P_c) of the plant. The elevation angle (θ) of each plant pixel (P_n) to the P_c was measured and averaged to present the circadian motion of the plant. (B) The 3D pixels of the representative plant recorded across five days were projected, and the tips (the farthest P_n to P_c) of one leaf are white-colored to show the trajectory of leaf motion. The daytime and nighttime corresponding to the tip positions are indicated. (C) Fourteen-day-old seedlings of the wild-type (Col-0), *bpc1-1 bpc2* (*bpc1,2*), and *bpc1-1 bpc2 bpc3-1* (*bpc1,2,3*) grown under long-day condition (16-h light/8-h dark, 80-100 μmol m^{-2} s^{-1} of white light) were monitored by recording the elevation angle at the indicated time under the day-night cycle. Data are mean ± S.E. (n = 7-9). The data of Col-0 are duplicated in the charts for comparison. White and black bars indicate the day and night periods, respectively.

hence reconstituted 2.5D images by incorporating the depth information [depth (z-axis); Figure 3A] obtained from the 3D pixels to the nearest pixels in the 2D image (*x-y* plane; Figure 3A). The constituted 2.5D images recorded from a five-day

period were projected to show leaf motion intuitively. The leaf tip point was labeled and showed a circadian trajectory by moving up and down during the daytime and nighttime respectively across circadian cycles (Figure 3B). We next manually selected the central point of the 2D plant correspondingly to the putative position of the apical meristem. The point was assigned with the lowest depth and used as the basal point (*Pc*; Figure 3A). *Pn* representing 2.5D pixels instituting the plant was used to calculate the elevation angle "*q*" relative to *Pc*, which generally characterized the plant motion (Figure 3A). The plant motion of *bpc1,2* and *bpc1,2,3* mutants was impeded as the elevation angle was significantly decreased (Figure 3C). The plant motion in *bpc1,2,3* was hampered more than that in *bpc1,2* (Figure 3C), suggesting that BPC1, 2, and 3 were additively required for circadian leaf motion.

The dawn and dusk clock components are oppositely regulated by class I BPCs

The impeded circadian motion shown by the *bpc1,2* and *bpc1,2,3* mutants has implied that the operation of the circadian clock is regulated by class I BPC. We next asked if the components of the circadian clock are affected by the mutations of BPCs. The representative dawn and dusk genes, *CCA1* and *EARLY FLOWERING4* (*ELF4*), were examined in the mutants. The long-day-entrained (16-h light/8-h dark) plants were transferred to the free-running condition for testing the clock oscillation. The representative genes oscillated in the mutants with altered amplitudes in the mutants (Figure 4). The peak of *CCA1* was slightly decreased in *bpc1,2* (Figure 4A, left panel) and further decreased in *bpc1,2,3* (Figure 2A, right panel). On the contrary, the peak of *ELF4* was slightly increased in *bpc1,2* and dramatically increased in *bpc1,2,3* (Figure 4B). GI is decreased in *bpc1,2* and is partially rescued in *bpc1,2,3* (Figure 4C). The effect of class I BPCs on clock components was consistent with that shown by the circadian motion (Figure 3C); class I BPCs affected the clock genes additively. However, class I BPCs positively and negatively functioned on *CCA1* and *ELF4*, respectively (Figure 4).

The robustness of the circadian clock oscillation can be reflected by the relative

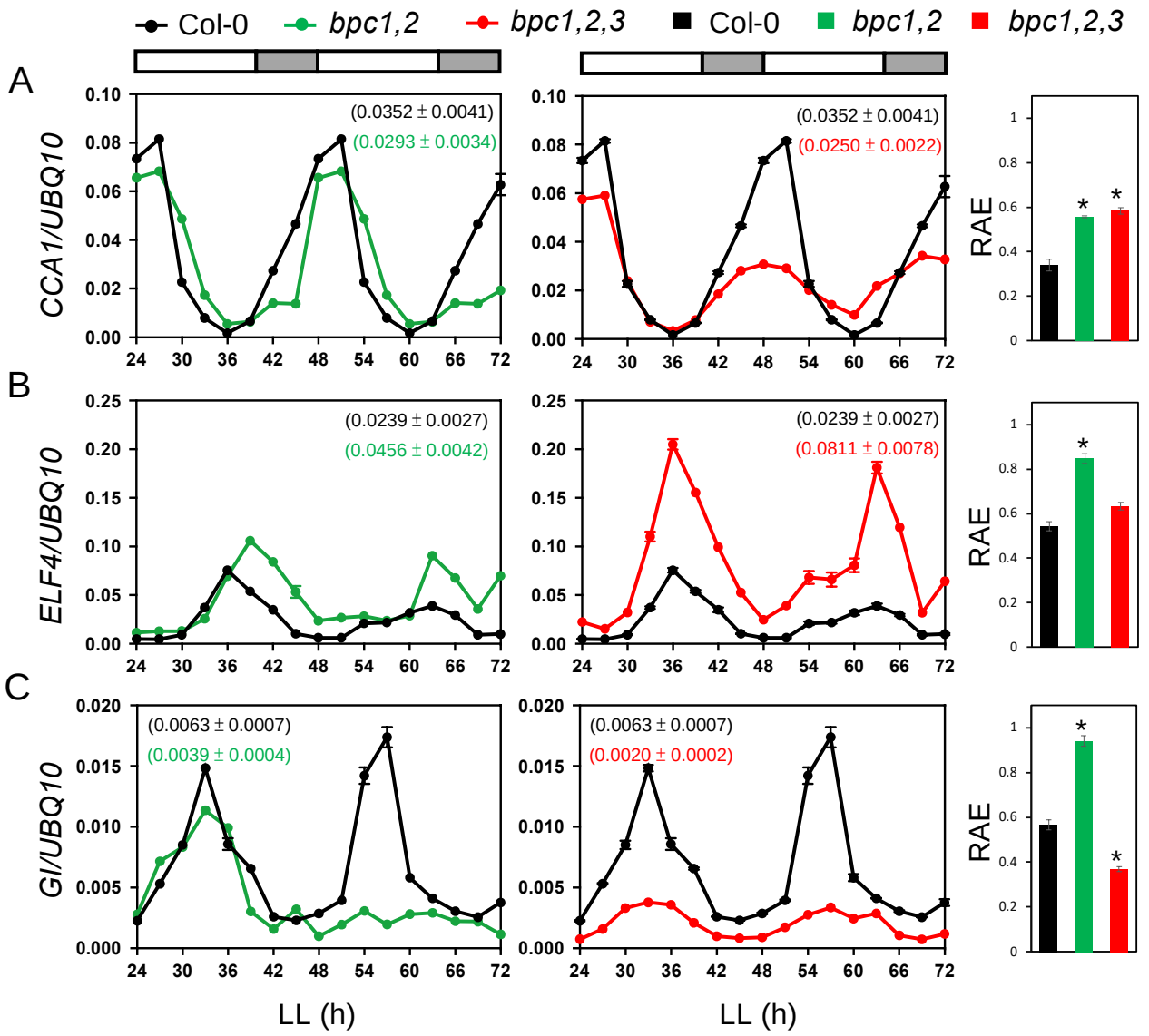

Figure 4 | The dawn and dusk genes are repressed and enhanced respectively in *bpc* mutants

Eighteen-day-old seedlings of the wild type (Col-0), *bpc1,2*, and *bpc1,2,3* grown under the long-day condition were transferred to the constant light (LL) and harvested every 3-h from LL24h to LL72h. The circadian clock operation was assessed in the harvested plants by real-time quantitative reverse-transcription PCR (RT-qPCR) assays. The arithmetic means of transcript levels in Col-0, *bpc1,2*, and *bpc1,2,3* over time are shown in black, green, and red parentheses, respectively. Data are mean ± S.E. (n = 51). The expression profiles and relative amplitude error (RAE) of *CCA1* (A), *ELF4* (B), and GI (C) were analyzed for presenting the clock oscillation. Data are mean ± S.E. (n = 3). White and gray bars indicated the subjective day and night periods, respectively. Asterisks indicate that the inducer treatment significantly altered the relative amplitude error (RAE) (Student's t-test; $P < 0.01$; n = 3).

amplitude error (RAE) of the gene profile. The RAE of *CCA1* profile was significantly enhanced in both *bpc1,2* and *bpc1,2,3* (Figure 4A), however, the RAE increases of *ELF4* and *GI* in *bpc1,2* were recovered and decreased by the mutation of *bpc3-1*, respectively (Figure 4B, C). The results of RAE analysis suggested that BPC1 and BPC2 sustained the persistence of evening genes via antagonizing BPC3, nevertheless, they sustained the morning gene through an unknown mechanism.

The *PRR* repressors of *CCA1* are enhanced in *bpc* mutants

We next extended our examination on the clock day genes, *PSEUDORESPONSE REGULATOR9* (*PRR9*), *PRR7*, *PRR5*, and *TOC1* (also known as *PRR1*) which repress *CCA1* consecutively from morning to night. All the repressors were increased slightly in *bpc1,2* and strongly in *bpc1,2,3* by comparing arithmetic mean of transcript levels over time (Figure 5A), suggesting that class I BPCs are required for the repression of *CCA1* repressors. Nevertheless, all the components are interlocked with each other directly or indirectly in the clock system. We could not rule out the possibility that class I BPCs directly activated *CCA1*. Therefore, two hypotheses were raised for further tests. First, class I BPCs directly activated *CCA1*. Second, class I BPCs activated *CCA1* indirectly by repressing *PRRs* (Figure 5B). Unfortunately, the oscillation of *PRRs* in our profiling did not show suitable RAEs for evaluating the effect of *bpc* mutations on the robustness of *PRRs*, particularly the RAEs of *PRR9* and *PRR5* (Figure 5A). A high-resolution and accuracy approach, such as promoter::luciferase reporter for the bioluminescence assay may improve our assays[31]

The overexpression of class I BPCs interferes with clock network

To test the role of class I BPCs in *CCA1* regulation, we applied the XVE-chemical inducible system [38] for the functional assays of BPC1 and BPC3. The synthetic LexA-VP16-ER (XVE) transcription factor driven by the constitutive promoter *G-box 10 to 35Smini -90* (*G10-90*) was used to trigger the expression of BPC1-EYFP-HA fusion protein

under *LexAop* control (Figure 6). Without the inducer estrogen, the XVE protein would be

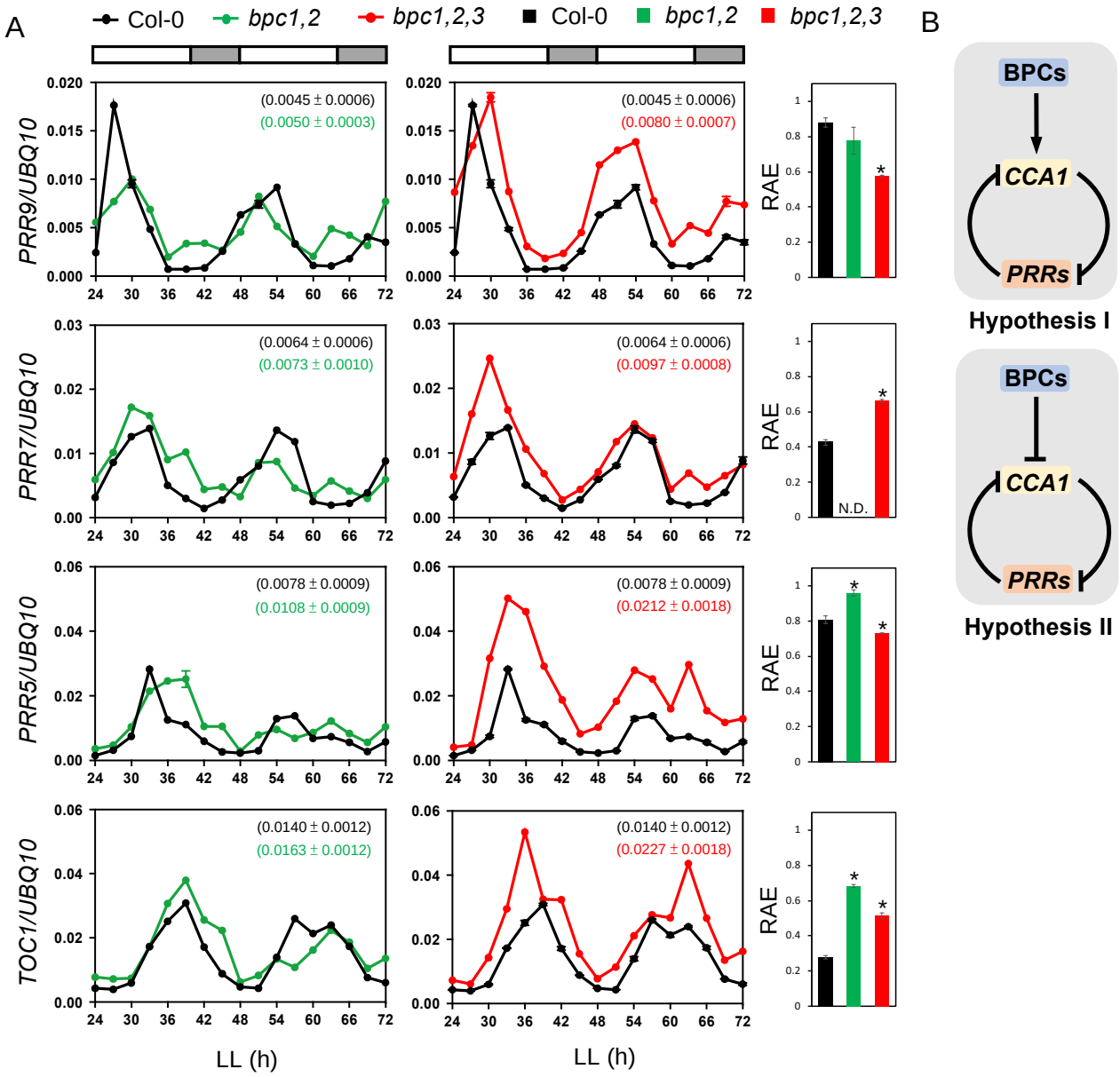

Figure 5 | The repressors genes of the *CCA1* are generally enhanced in *bpc* mutants

(A) The expression of clock components *PRR9/7/5/TOC1* that consecutively repress *CCA1* from day to night was profiled under the free-running condition in Col-0, *bpc1,2*, and *bpc1,2,3*. The genes were profiled as described in Figure 1. (B) Two hypotheses were proposed for assessing the role of class I BPCs in the regulation of the circadian clock. Hypothesis I: BPCs directly activate *CCA1*. Hypothesis II: BPCs activate *CCA1* via the repression of its repressors, *PRRs*. The arithmetic mean of transcript levels in Col-0, bpc1,2, and bpc1,2,3 over time is shown in black, green, and red parentheses, respectively. Data are mean ± S.E. (n = 51).

trapped in the cytoplasm by the endogenous heat shock proteins (HSPs) which bind to the ER ligand binding domain (LBD, Figure 6). After estrogen treatment, the HSP-trapped XVE

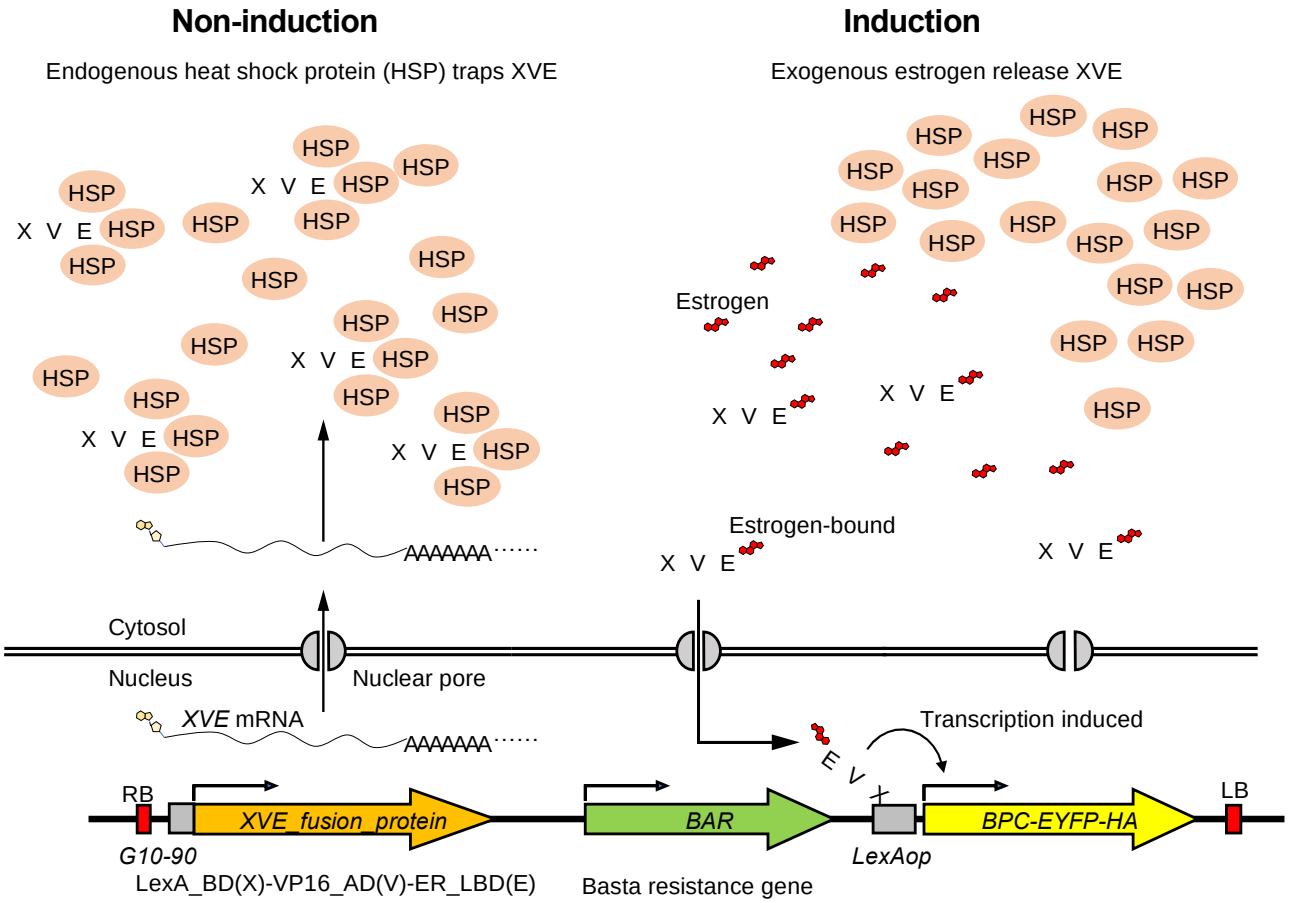

Figure 6 | Functional examination for class I BPCs under the chemical inducible XVE system

The overexpression of the class I BPC proteins are controlled by the chemical-inducible system. The transcription of the synthetic transcription factor XVE composing LexA operator binding domain (LexA_BD; X), VP16 activation domain (VP16_AD; V), and estrogen ligand binding domain (ER_LBD; E) is driven by the constitutive promoter *G10-90*. Under the non-induction condition, translated XVE protein would be trapped in the cytoplasm by the endogenous heat shock protein (HSP), which forms protein complexes via binding to the ER_LBD of XVE. By treating exogenous estrogen, ER_LBD binds to the estrogen, and HSPs are dissociated and release XVE. ER_LBD is a simultaneous strong nuclear localization signal that brings XVE into the nucleus. In the nucleus, the LexA_BD targets the *LexA* operator (*LexAop*), and the VP16_AD recruits RNA polymerase II to induce the transcription of *BPC-EYFP-HA*. *BAR*, the BASTA herbicide-resistant gene for transgenic plant selection. RB and LB, the right and left border sequence of the transfer DNA (T-DNA).

would bind to estrogen and is released from HSPs, and the nuclear transportation of XVE is thus triggered (Figure 6). In the nucleus, the LexA-binding domain of XVE targets *LexAop* and activates the downstream gene transcription by the VP16 activation domain (Figure 6). Consequently, we would control the overexpression of class I BPC1 by adding estrogen to the transgenic plants selected by using an herbicide marker (BAR, Figure 6) and investigate the instant effect of the BPC overexpression. The 18-day-old transgenic plants harboring the inducible system of BPC1-EYFP-HA or BPC3-EYFP-HA were

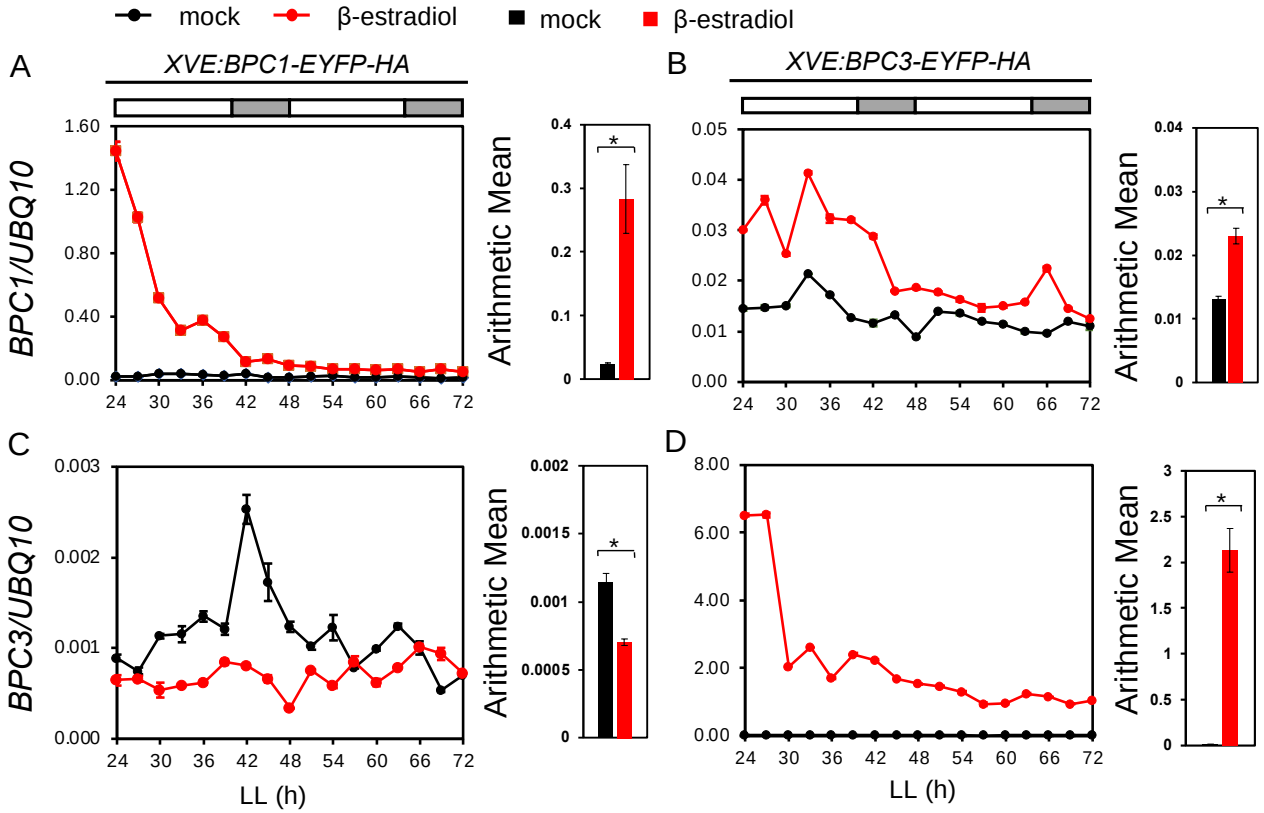

Figure 7 | BPC1 and BPC3 are negative and positive regulators of each other respectively

Eighteen-day-old seedlings of transgenic lines of *BPC1-EYFP-HA* and *BPC3-EYFP-HA* grown under long-day conditions were transferred to the constant light and treated with 0 μM (mock) or 50 μM β-estradiol under the free-running condition. The RT-qPCR assays for *BPC1* and *BPC3* profiling under the indicated conditions were conducted as described in Figure 4. (A, D) The enhancing effect of the estrogen treatment on the *BPC1* and *BPC3* transcripts was validated. The effect of BPC1 on the expression of *BPC3* (B) and that of BPC3 on the expression of *BPC1* was assessed. The arithmetic mean of transcript levels over time was calculated for estimation of gene expression level.

entrained under the long-day condition and transferred to the free-running condition with (50 μM β-estradiol) or without (0 μM) estrogen treatment.

We first profiled the induction of *BPC1* or *BPC3* transcript level under the induction. The estrogen treatment has enhanced the *BPC1* or *BPC3* transcript successfully in the corresponding transgenic lines (Figure 7A, D). Furthermore, the transcript level of endogenous *BPC3* was repressed by BPC1 (Figure 7C), consistent with the genetic effect shown by the *bpc1* mutation [12]. Besides, the endogenous *BPC1* was increased by BPC3 (Figure 7B), consistent with the positive effect of BPC3 on *BPC1* shown in a previous study [14]. Taken together, the inducible lines carrying the BPC1-EYFP-HA and BPC3-EYFP-HA are functional.

As BPC3 is a cryptic repressor of *CCA1* [14], we also included BPC3 as a positive control for validating our functional assays for BPC1. Under the induction of BPC1 overexpression, the expression of *CCA1* was significantly repressed (Figure 8A), the repression conducted by BPC3 (Figure 8B). The repression of *CCA1* conducted by the

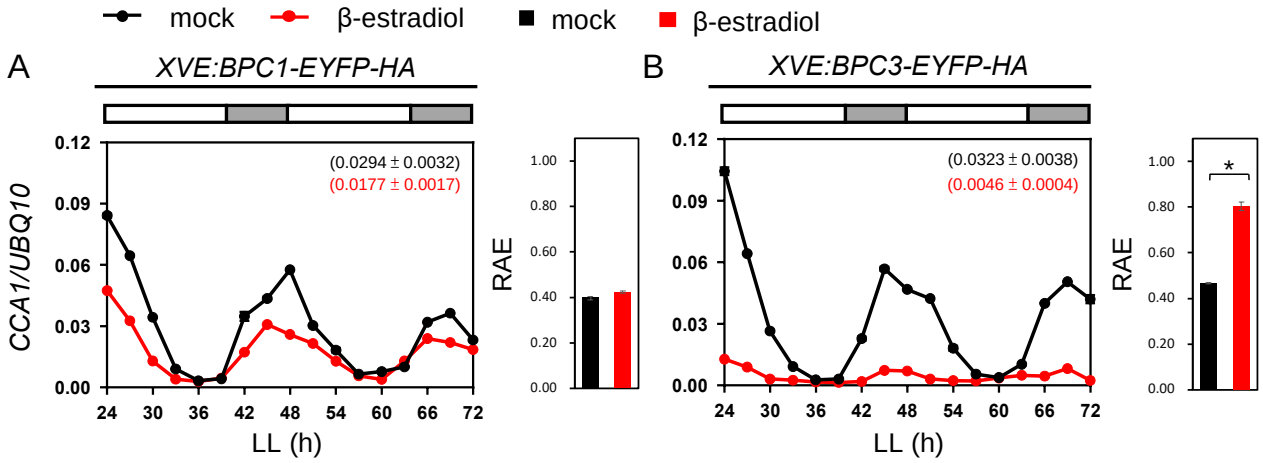

Figure 8 | *CCA1* is repressed by the overexpression of class I BPCs

The effect of class I BPCs (BPC1 and BPC3) on the expression of *CCA1* was assessed as described in Figure 7. Asterisks indicate that the inducer treatment significantly altered the relative amplitude error (RAE) (Student's t-test; $P < 0.01$; $n = 3$). The arithmetic means of transcript levels under BPC1 and BPC3 induction over time are shown in black and red parentheses, respectively. Data are mean ± S.E. ($n = 51$).

overexpression of BPC1 and BPC3 has suggested that hypothesis I, which hypothesized that class I BPCs directly activate *CCA1* cannot be right (Figure 5B).

It can be noted from the RAE values that BPC1 overexpression barely affected *CCA1* rhythmicity, while BPC3 overexpression greatly diminished it.

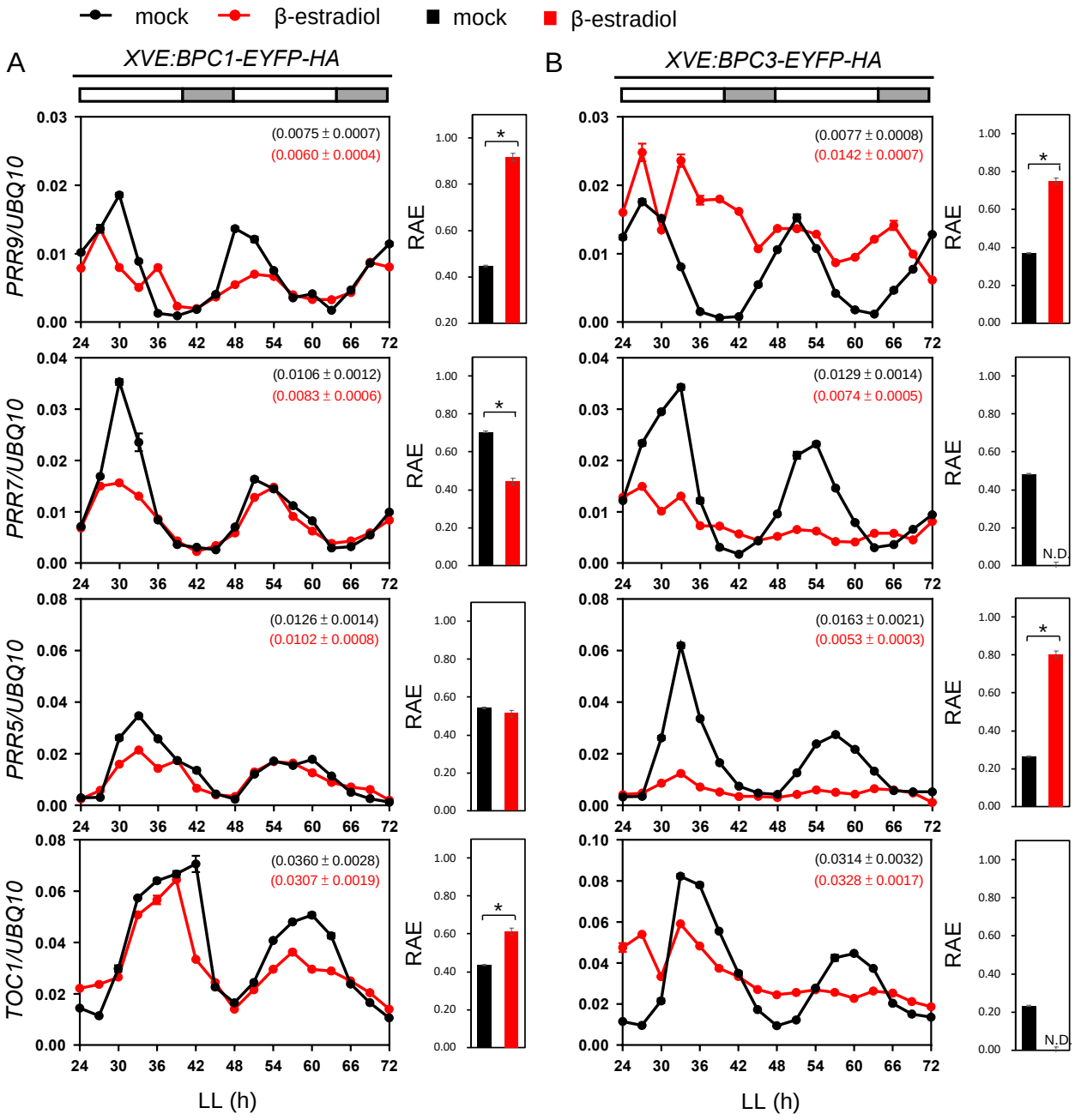

Figure 9 | *PRRs* are impeded by the overexpression of class I BPCs

The effect of BPC1 (A) and BPC3 (B) on the expression of *PRRs* was assessed as described in Figure 7. The arithmetic means of transcript levels under BPC1 and BPC3 induction over time are shown in black and red parentheses, respectively. Data are mean ± S.E. (n = 51).

We next tested if the induction of BPC1 and BPC3 overexpression would repress *PRRs*. As shown in Figure 9A, the expression of *PRRs* was slightly repressed by the induction of BPC1 overexpression (Figure 9A). *PRR7* and *PRR5* were strongly repressed by the induction of BPC3 overexpression (Figure 9B). However, *PRR9* and *TOC1* were strongly and slightly enhanced by BPC3 induction, respectively (Figure 9B). Overall, BPC3 drastically impeded the oscillation of *PRRs* (Figure 9B). This indicates that hypothesis II stands that the class I BPCs indirectly activate *CCA1* by repressing *PRRs* (Figure 5B). Again, the oscillation of *PRRs* was not suitable for the RAE analysis here (Figure 9).

We also examined the expression of dusk clock genes, namely *ELF4* and *GI*, in the class I BPC induction line. As shown in Figure 10, *GI* expression was ever so slightly increased under BPC1 overexpression (Figure 10A), whilst under BP3 overexpression, the *GI* expression peaks were damped with an increase of the arithmetic mean of transcript levels over time (Figure 10B). *ELF4* on the other hand, was strongly repressed by BPC1 overexpression (Figure 10A), and even more significantly downregulated by BPC3 overexpression (Figure 10B).

As for the RAE analysis results, the RAE values in both *GI* and *ELF4* was decreased under BPC1 overexpression. The RAE value alterations under BPC3 overexpression, however, were drastically different: the *GI* oscillation was arrhythmic (RAE was not detected) and the RAE of *ELF4* was markedly enhanced. It can be suggested that BPC1 contrubutes to the stability of evening gene rhythmicity while BPC3 disrupts the circadian pattern of dusk clock genes.

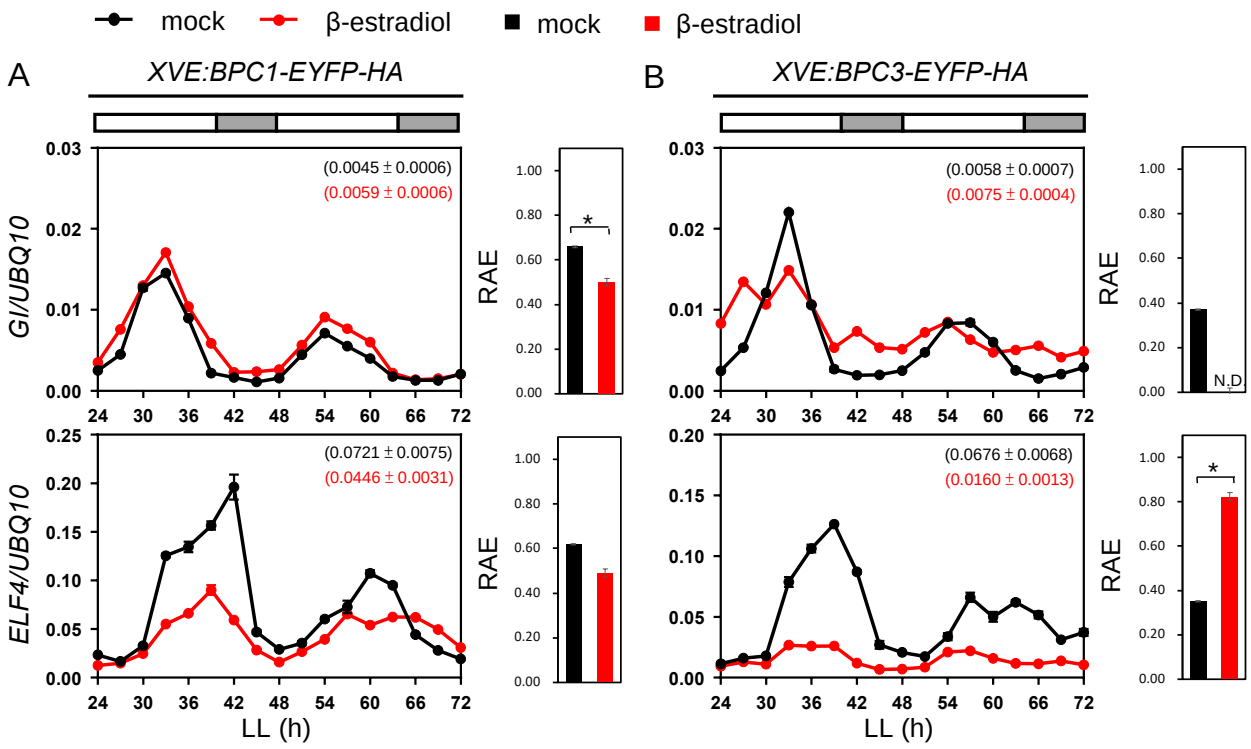

Figure 10 | Evening genes are impeded by the overexpression of class I BPCs

The effect of BPC1 (A) and BPC3 (B) on the expression of *GI* and *ELF4* was assessed as described in Figure 7. The arithmetic means of transcript levels under BPC1 and BPC3 induction over time are shown in black and red parentheses, respectively. Data are mean ± S.E. (n = 51).

Leaf edge controlling genes, class II *TCP*s, are impeded by class I BPC overexpression

It was observed that in class II *tcp* mutants, the leaf edge becomes curly and chevroned [39]. We were curious whether class I BPCs play a role in regulating class II TCP expression. As shown in Figure 11, BPC1 and BPC3 both repress class II TCPs, with BPC3 clearly being the stronger repressor of *TCP3*, *TCP4*, *TCP10*, and *TCP17*, but not *TCP5* and *TCP13* (Figure 11A). According to arithmetic mean calculations, BPC3 barely affected *TCP5* but slightly impeded *TCP13* (Figure 11B). BPC1 did not affect *TCP3* and *TCP4* significantly, increasing *TCP5* and decreasing *TCP10*, *TCP13*, and *TCP17* (Figure 11B).

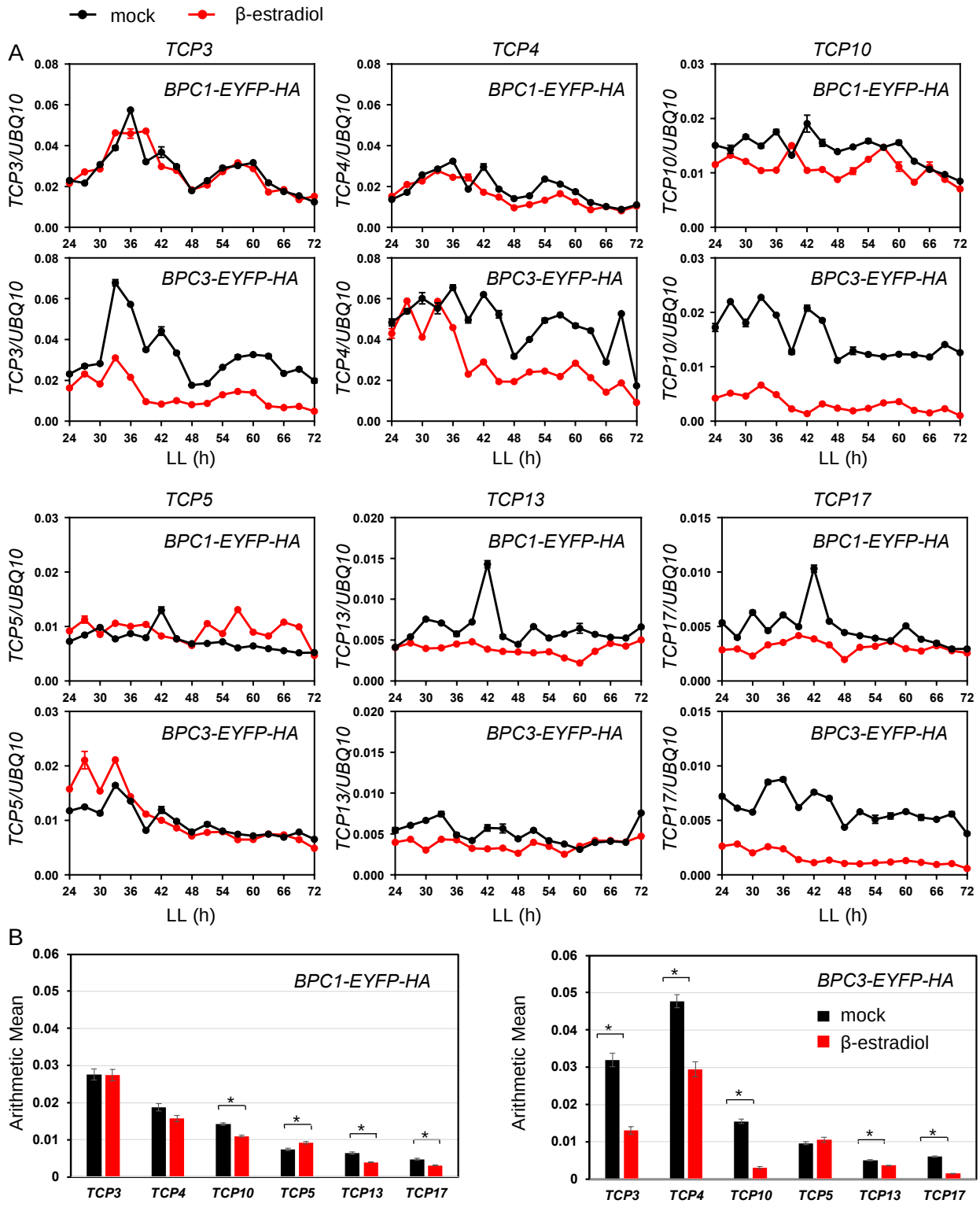

Figure 11 | Class II TCPs controlling leaf edge development are impeded by the overexpression of class I BPCs

(A) The effect of class I BPCs on the expression of class II *TCPs* was assessed as described in Figure 7. (B) The arithmetic mean of transcript levels over time was calculated for estimation of gene expression level.

BPC2 exhibits similar functions

BPC2 is known to be structurally akin to BPC1. To determine if they function in a similar fashion, we harvested seedlings ever 3 hours starting from the 24th to 48th hour of constant light conditions and conducted RT-qPCR tests. In the control and BPC2 induced lines, the expression of BPC2 itself, BPC3, representative clock gene, *CCA1*, and leaf developmental gene, *TCP4*, are profiled. As shown in Figure 12, the BPC2 induction was validated (Figure 12A); the expression of *BPC3*, *CCA1*, and *TCP4* was impeded by BPC2 overexpression (Figure 12B, C, D). The data obtained suggested that BPC2 does have overlapping functions with its analogue, BPC1.

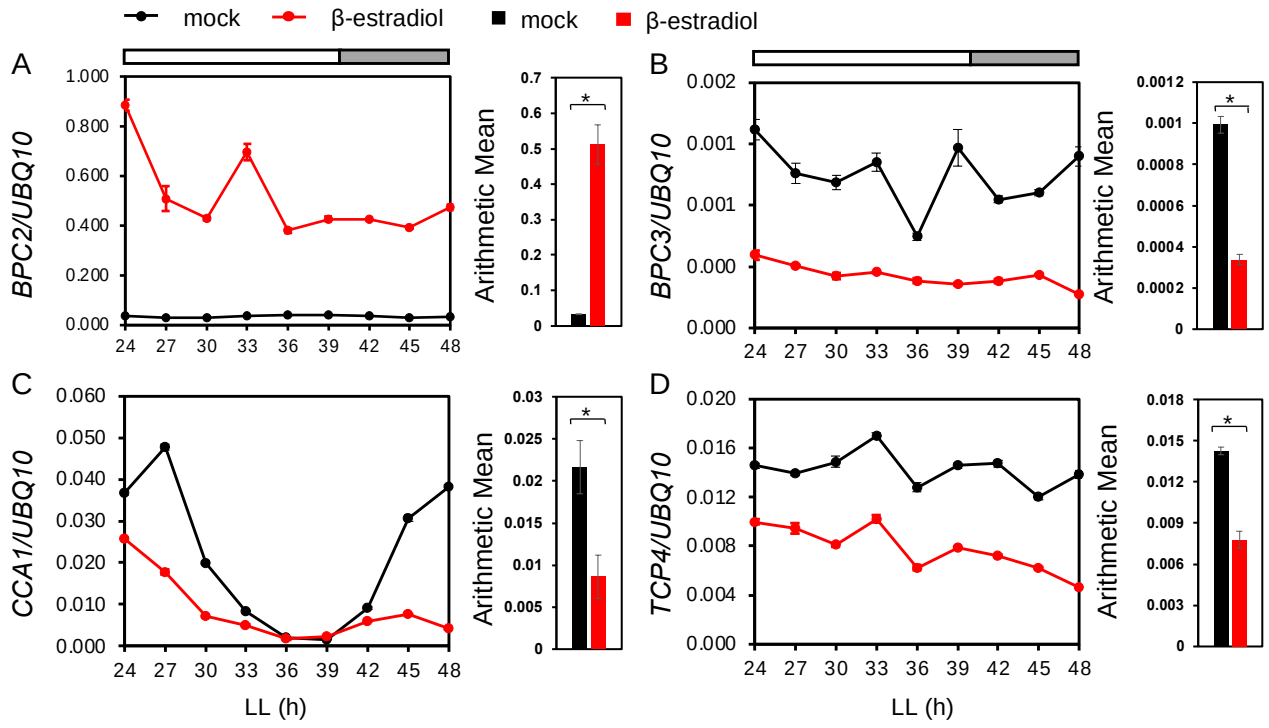

Figure 12 | BPC2 functions consistently with other class I BPCs

The induction of BPC2 was validated (A). The effect of BPC2 on the expression of (B) *BPC3* (C) *CCA1* and (D) *TCP4* was assessed as described in Figure 7. The arithmetic mean of transcript levels over time was calculated for estimation of gene expression level.

The induction extent of class I BPCs in transgenic lines is determined

To validate the induction of class I BPCs in our transgenic plants, we examined the protein induction by using anti-HA antibody with the western blot assay. The protein signals

of EYFP-HA-fused BPCs were semi-quantified and normalized to the amount of coomassie blue stained total extracted protein. As shown in Figure 13, the corresponding BPCs were detected in the transgenic lines under the induction. Nevertheless, the induction extent of BPC3-EYFP-HA line was significantly greater than that of BPC1-EYFP-HA and BPC2-EYFP-HA lines (Figure 13), suggesting that the BPC repressive effect might correlate with the induction extent.

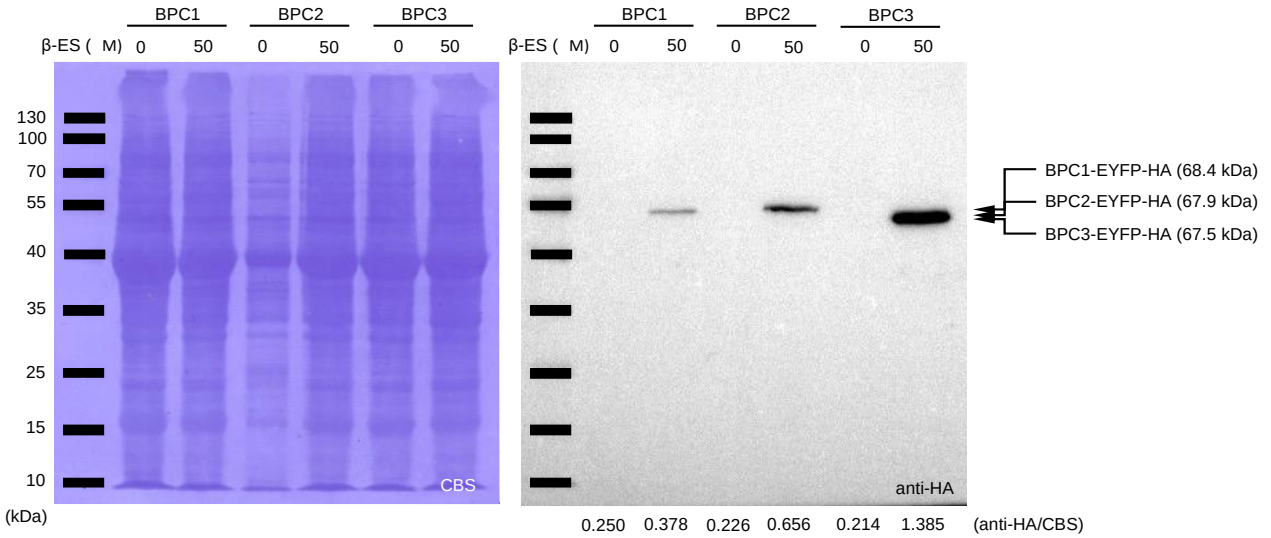

Figure 13 | The induction of class I BPCs in the transgenic lines was validated by western blot analysis

The transgenic lines of BPC1-EYFP-HA, BPC2-EYFP-HA, and BPC3-EYFP-HA driven by XVE induction system were treated with 0 or 50 μM of 17-β-estradiol and were collected for protein preparation after 2-day treatment. The protein samples were analyzed by using anti-HA antibody in a western blot assay. The HA signals were quantitated by using ImageJ 1.52 software. The Coomassie Blue staining (CBS) signal of each sample was used for signal normalization (anti-HA/CBS) to show the induction level.

Discussion

It is known that the plant leaf movement is controlled by the circadian clock [40,41]. Moreover, in the plants with growth defects, the leaf movement is usually hampered [41-43]. We observed that in class I bpc mutants also have such growth problems, and hence we examined the leaf movement of these plants, obtaining the same hampered results. In our clock gene profiling results, the impeded plant motion is highly correlated with the damped

expression of *CCA1* (Figure 3C and 4A), suggesting that class I BPCs might be involved in the connection between leaf movement and clock oscillation.

The qPCR results are summarized in Figure 14. Throughout our experiments, we observed that BPC3 is usually a much stronger repressor than BPC1. Take *CCA1* for an example, whilst the peaks and valleys in clock gene mRNA expression can still be identified though hampered under BPC1 overexpression, the expression curve under BPC3 overexpression is significantly disfigured, with no obvious rhythmic oscillations. The same goes for *PRR7*, *PRR5*, *TOC1*, and *ELF4*. As for *GI*, the effects BPC3 has on it is also larger. We hypothesized that such results may be due to two reasons: (1) The protein expression of BPC3 is materially greater than that of BPC1 due to unknown post-transcriptional regulatory pathways. (2) Through sequence analysis by past research, it was found that whilst all BPCs have a conservative sequence of 5 cysteines at the C terminal serving as a DNA binding site, the N terminals have different arrangements of motifs which may lead to variations in binding affinity or protein recruitment. Through conducting western blot analysis, we suggest that reason 1 may be the more explicit explanation.

Through the mutant line experiments, we proposed 2 hypotheses as to why deficiency in BPCs lower *CCA1* and rises *PRRs* mRNA expression: (1) BPCs directly stimulate *CCA1* transcription, which represses *PRRs* according to past research (2) BPCs repress *PRRs*, repressors of *CCA1*, and thus indirectly stimulate *CCA1* transcription. By conducting BPCs overexpression, we suggested that hypothesis 1 is likely to be false since generally both BPC1 and BPC3 have repressor activities on *CCA1* and *PRRs*. Interestingly, there are two exceptions: BPC3 is a *PRR9* and *TOC1* stimulator.

Both the mutant and inducible lines tell us that class I BPCs are repressors of the evening gene, *ELF4*, which goes to show that BPCs regulate the circadian clock by directly repressing evening genes and indirectly affecting the morning genes (Figures 4A, 4B, 8, and 10). Nevertheless, the regulation of *GI* is specifically conducted by BPC3 (Figure 10). When *BPC1* and *BPC2* expression decreases, *BPC3* is freed of inhibition, as BPC1 and BPC2

have overlapping function to inhibit *BPC3* (Figure 7C, 12B). Therefore, *GI* is repressed (Figure 4C). If we also mutate *BPC3*, *GI* expression can be rescued (Figure 4C). In the inducible line, we also obtained the same results: BPC1 overexpression further damps the already low *BPC3* expression, and so *GI* expression is enhanced slightly (Figure 10).

Examining class I BPCs' regulation of *TCP3*, *TCP4*, *TCP10*, *TCP5*, *TCP13*, *TCP17*, we have a clearer understanding of the relationship between BPC and TCP family (Figure 11). Other members in the TCP family, namely TCP20 and TCP22, are stimulators of the circadian clock genes [32] while class I BPCs tend to be repressors [14]. Class I BPCs also indirectly impact leaf edge development by repressing certain *TCPs*. Aside from BPCs, class II *TCPs* that control leaf edge development are also regulated by miR319 [39,44], suggesting that class II *TCPs* can be regulated by both transcriptional and post-transcriptional means.

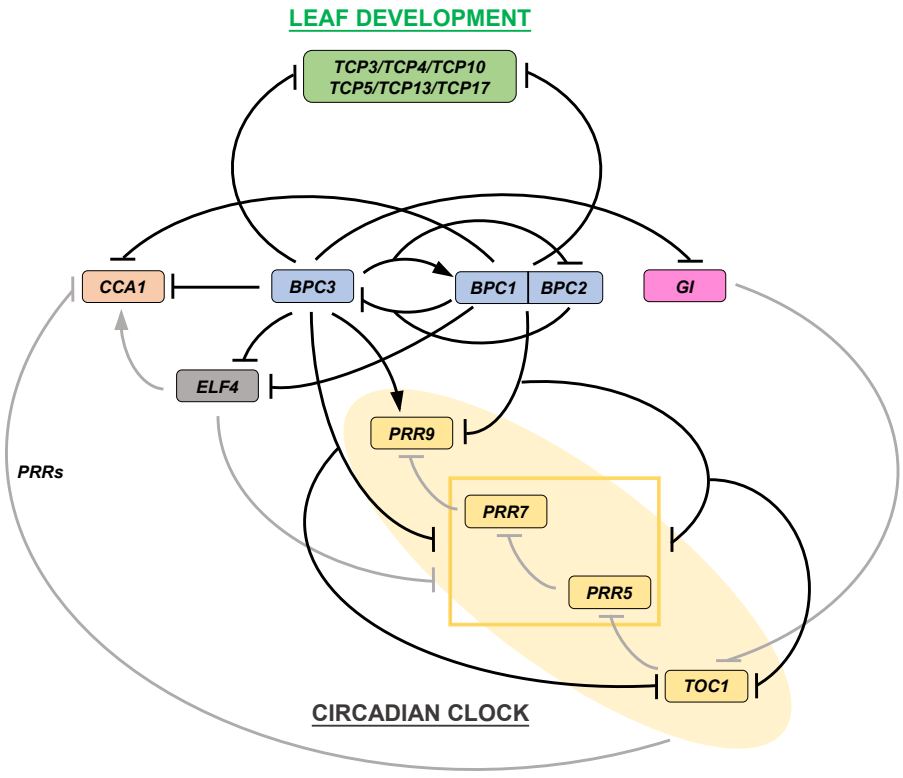

Figure 14 | Regulatory model of class I BPC

This diagram serves as a summary of the RT-qPCR results. Results from past research are depicted in grey lines; black lines are our results obtained by mutant and inducible lines.

Conclusion

Our work sheds light on the importance of class I BPCs, which is known to greatly affect plant growth and development. Prior to our research, BPC1 was only viewed as a repressor for BPC3. We reveal that class I BPCs play as a hub between leaf development and the circadian clock. Particularly, the impacts of BPC1 on the circadian clock became more vivid: not only does it act as an antagonist of BPC3, but it also plays a role in repressing clock genes from morning to night. Our study also inspires significant biological questions to be further researched. The worthwhile step will be to identify the entry genes targeted by class I BPCs in the circadian clock to decipher the mechanism that equilibrates the *bpc* mutant impact to run a compromised oscillator.

Limitations and Future Work

In the future, we could adjust the β-estradiol concentration with the aim of achieving identical degrees of class I BPCs for our XVE-transgenic lines. Moreover, more precise means, such as enzyme-linked immunosorbent assays (ELISAs), to measure the class I BPC levels in transgenic lines would be helpful to accurately quantify their effectiveness as transcriptional factors. Additionally, we cannot be sure whether the stimulatory activity is direct or indirect through repressing other repressors. It is possible that because *PRR7* and *PRR5* are repressed by class I BPCs, they cannot successfully lower *PRR9's* expression at noon, and hence a plateau is formed. In addition, the transcript levels of target genes in our RT-qPCRs are the steady-state results of the combination of transcriptional and post-transcriptional regulations on the genes. To be clear about the mechanism, we will have to conduct a bioluminescence assay by using a luciferase reporter driven by promotors of target genes for studying the transcriptional mechanism. In theory, if class BPCs instantly boost or inhibit target gene expression upon overexpression rather than waiting 24 hours for an equilibrium to be reached, we can conclude that class I BPCs are stimulators or repressors since more complex pathways require more time to be effective. Moreover,

chromatin immunoprecipitation (ChIP) assays can be helpful at providing evidence whether class I BPCs associate directly with target gene promoters. Furthermore, the interlocking complex constituted by clock genes have been described by using the mathematic simulation [45]. Looking forward, Class I BPCs can be also incorporated into a mathematic model to decipher their dynamic effects on clock components.

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【評語】060005

一、本研究藉由比較雙/三突變或過量表現植株中，葉片運動角度或自由運轉周期的改變，以及藉由 RT-qPCR 檢測重要蓋日韻律調節基因(例如 CCA1 andPRRs)表現量的增減，確定 BPC 涉及植物生理時鐘的調節。

二、此研究目標明確，且實驗過程及結果完整度高。

三、過量表現植株以 BASTA 篩選後，如何確定 BPC 有表現仍待釐清。